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Removal of Pex3p Is an Important Initial Stage in Selective Peroxisome Degradation in *Hansenula polymorpha**

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Selective degradation of peroxisomes (macropexophagy) in *Hansenula polymorpha* involves the sequestration of individual organelles to be degraded by membranes prior to the fusion of this compartment with the vacuole and subsequent degradation of the whole organelle by vacuolar hydrolases. Here we show that Pex3p, a peroxisomal membrane protein essential for peroxisome biogenesis, escapes this autophagic process. Upon induction of macropexophagy, Pex3p is removed from the organelle tagged for degradation prior to its sequestration. Our data indicate that Pex3p degradation is essential to allow the initiation of the organellar degradation process. Also, in a specific peroxisome degradation-deficient (*pdd*) mutant in which sequestration still occurs but the vacuolar fusion event is disturbed, the turnover of Pex3p is still observed. Taken together, our data suggest that degradation of Pex3p is part of the initial degradation machinery of individual peroxisomes.

Peroxisomes are important organelles that play a role in various metabolic processes in eukaryotes. In fungi, they are predominantly involved in the oxidative metabolism of the carbon and/or nitrogen source used for growth. Characteristically, during cultivation on compounds that require the function of peroxisomal enzymes, the organelles rapidly develop and contain the key enzymes involved in the metabolism of the specific growth substrate. The opposite also occurs. When cells grown at peroxisome-inducing conditions are placed in fresh media in which these functions are no longer required for growth, the organelles are rapidly and selectively degraded by an autophagic process, also termed pexophagy (1–2).

In *Hansenula polymorpha*, selective degradation of peroxisomes is induced when methanol-grown cells are shifted to fresh glucose- or ethanol-containing media. This process involves three morphologically distinct steps, namely (i) sequestration of the organelle to be degraded from the cytosol by various membranous layers, (ii) the fusion of the outer membranous layer of the sequestered compartment with the vacuolar membrane followed by (iii) the degradation of the organel-

lar components by vacuolar hydrolases (3). This process has been designated macropexophagy.

In the related species *Pichia pastoris*, a similar process takes place after a shift of cells from methanol to ethanol. However, when glucose is used to induce pexophagy, an alternative degradation pathway is initiated termed micropexophagy. The hallmark of this process is that clusters of peroxisomes are engulfed by the vacuole (4).

Recently, we observed that the mechanisms of peroxisome biogenesis and selective degradation in *H. polymorpha* use factors in common. We demonstrated that Pex14p, a crucial component in matrix protein import, is also essential for selective peroxisome degradation in *H. polymorpha* (5, 6). Although the molecular mechanisms of the dual function of Pex14p in the two oppositely directed processes are still unresolved, we showed that the information that controls degradation resides in the N terminus of the protein (6).

Here, we show that a second peroxin Pex3p, which is essential for peroxisome biogenesis and maintenance (7), also plays a role in macropexophagy in *H. polymorpha*. At present, a consensus exists that upon induction of macropexophagy in yeast, the whole organelle is degraded by vacuolar hydrolases. In this paper, we demonstrate that Pex3p escapes this process and is degraded prior to uptake of the organelle by the vacuole. We have interpreted our data in that degradation of Pex3p is an essential early step in selective degradation of individual peroxisomes by macropexophagy.

MATERIALS AND METHODS

Organisms, Cultivation Media, and Conditions—The *H. polymorpha* strains used in this study are described in Table I. *H. polymorpha* cells were grown in batch cultures at 37 °C on (a) selective minimal medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco) supplemented with 1% (w/v) glucose, (b) rich medium containing 1% (w/v) yeast extract, 1% (w/v) peptone, and 1% (w/v) glucose, or (c) mineral medium (8) supplemented with 0.5% (w/v) glucose, 0.5% (v/v) methanol, or a mixture of 0.1% (v/v) glycerol and 0.5% (v/v) methanol as carbon source together with 0.25% (w/v) ammonium sulfate as nitrogen source. When required, leucine (30 µg/ml) was added. During protease inhibition experiments, the proteasome inhibitor MG-132 was added to the culture to a final concentration of 50 µM (9).

For analysis of peroxisome degradation, cells were extensively pre-cultivated on mineral medium containing glucose and shifted to mineral medium containing methanol to induce peroxisome proliferation. 0.5% glucose was added to induce selective peroxisome degradation. *Escherichia coli* DH5α (10) was used for plasmid amplification and was grown on LB medium supplemented with the appropriate antibiotics.

Molecular Techniques—Standard recombinant DNA techniques (10) and transformation of *H. polymorpha* was performed as described previously (11). Restriction enzymes and biochemicals were obtained from Roche Molecular Biochemicals and used as detailed by the manufacturer.

Construction of the PEX3.GFP Strain—An *H. polymorpha* wild type strain in which the expression of the hybrid gene *PEX3.GFP* is regulated by the endogenous *PEX3* promoter (*P*_{PEX3}) was constructed as

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TABLE I
H. polymorpha strains used in this study

Strain	Relevant properties	Reference
WT NCYC495	<i>leu1.1</i>	16
<i>pex3::P_{PEX3}Sc_{PEX3}</i>	<i>PEX3</i> deletion strain containing <i>S. cerevisiae PEX3</i> under control of the endogenous <i>H. polymorpha PEX3</i> promoter.	12
<i>pdd1</i>	<i>PDD1</i> deletion strain.	12
<i>pdd2</i>	<i>pdd2-4</i> (peroxisome degradation-deficient mutant).	15
<i>pex14::P_{AOX}PEX5^{mc}</i>	<i>PEX14</i> deletion strain containing multiple copies of the <i>PEX5</i> overexpression cassette.	17
<i>PEX14-ΔN64</i>	<i>PEX14</i> deletion strain expressing a truncated <i>PEX14</i> gene that encodes Pex14p lacking the first 64 N-terminal amino acids.	6
PEX3-GFP	WT expressing a hybrid gene that encodes GFP fused to the C terminus of Pex3p. The hybrid gene is under control of the <i>PEX3</i> promoter.	This study

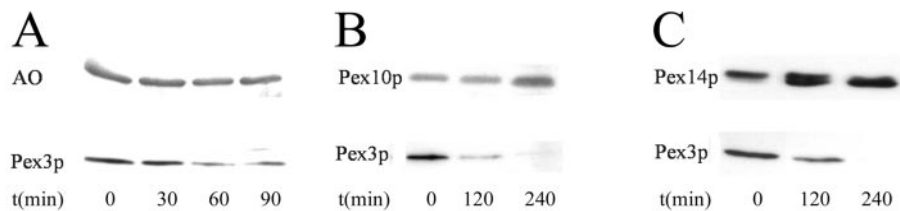


FIG. 1. **Pex3p degradation in *H. polymorpha* strains affected in selective peroxisome degradation.** A–C, Western blots showing the decrease in Pex3p protein levels under conditions that other peroxisomal proteins are not degraded in *H. polymorpha pdd2* (A), *Δpex14::P_{AOX}PEX5^{mc}* (B), and *PEX14-ΔN64* (C) cells. Cells were grown to the mid-exponential growth phase on glycerol-methanol containing media to induce peroxisome proliferation. Glucose was added to the cultures at *t* = 0 min. Samples were taken at regular time intervals after the addition of glucose. Samples corresponding to equal volumes of the cultures were loaded per lane. Western blots were decorated using α-Pex3p, α-Pex10p, α-Pex14p, or α-AO antibodies. In all three experiments, Pex3p diminishes in time, whereas other peroxisomal proteins are not degraded. The Pex14p blot in C shows two bands; the upper one represents phosphorylated Pex14p (38).

follows: first, a 7.2-kb *NotI-MluI* (both Klenow-treated) fragment of pFEM152 (11) was self-ligated, resulting in pHOR46. This plasmid contains only the 3'-end of *PEX3* encoding codons 157–457 fused to *GFP*. Subsequently, *H. polymorpha* NCYC495(*leu1.1*) cells were transformed with *StuI*-linearized pHOR46, and transformants were selected for leucine prototrophy. Correct integration into the *PEX3* locus was confirmed by Southern blot analysis (data not shown). A transformant containing a single copy integration was selected for further analysis and designated *PEX3.GFP*.

Biochemical Methods—Crude extracts from trichloroacetic acid-precipitated *H. polymorpha* cells were prepared as described previously (9). SDS-PAGE and Western blotting were performed by established procedures. Western blots were decorated using a chemiluminescent Western blot kit (Roche Molecular Biochemicals) and polyclonal antibodies against *H. polymorpha* alcohol oxidase (AO), Pex3p (7), Pex10p (12), and Pex14p (13).

Electron Microscopy—Whole cells were fixed and prepared for electron microscopy as described previously (7). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific polyclonal antibodies against *H. polymorpha* AO and gold-conjugated goat anti-rabbit antibodies (7).

RESULTS

Degradation of Pex3p in *pdd* Mutants—In general, selective peroxisome degradation in *H. polymorpha* is monitored by following the level of one of the major matrix enzymes: AO, dihydroxyacetone synthase, or catalase. In a conventional degradation experiment using this organism, the level of AO activity and protein decreases to approximately 10–20% of the original value within the first 4 h after the shift (3, 14, 15). Comparable observations have been made for peroxisomal membrane proteins. However, in the course of our experiments, we noticed that in cells of specific *H. polymorpha* mutants, which are impaired in selective peroxisome degradation (*pdd* mutants) (15), the peroxisomal membrane protein Pex3p nevertheless is degraded upon the addition of glucose. An example of this is shown in Fig. 1A. After a shift of methanol-grown *pdd2* (15) cells to glucose-containing medium, AO protein is not degraded as expected. However, in the same cells, the level of Pex3p is drastically reduced within a time interval of 90 min. (Fig. 1A).

Degradation of Pex3p in *pex14* Mutant Strains—To further analyze this phenomenon, we analyzed cells that lacked the membrane protein Pex14p. As shown before, Pex14p is essential for peroxisome biogenesis, and normal peroxisomes are absent in *PEX14* deletion strains (*pex14*) (13). However, in *H. polymorpha pex14* cells, peroxisome development can be restored by the overproduction of the PTS1 receptor, Pex5p (17). Also, because of the absence of Pex14p, these organelles are no longer susceptible to selective degradation (5). After a shift of methanol-induced *H. polymorpha pex14* cells that overproduce Pex5p (*pex14::P_{AOX}PEX5^{mc}*) to glucose-containing medium, Pex3p is again rapidly degraded, whereas Pex10p, an integral component of the peroxisomal membrane remained virtually unaffected (Fig. 1B). Essentially, similar results were obtained using cells producing a truncated form of Pex14p of which the first 64 amino acids are deleted (*PEX14-ΔN64*), and for that reason, they cannot degrade peroxisomes (Fig. 1C). Therefore, these results are in line with the assumption that the degradation pathway of Pex3p differs from that of other peroxisomal proteins upon the induction of selective peroxisome degradation in *H. polymorpha*.

Selective Peroxisome Degradation in WT Cells at Different Growth Stages—In previous experiments on WT *H. polymorpha* cells, the phenomenon of an alternative rapid Pex3p degradation pathway was less evident (14). We reasoned that this might be related to microbody numbers, and thus, to the growth stage of the cells. It is well documented that the cells of *H. polymorpha* that are in the early exponential growth phase on methanol generally contain a single peroxisome and that the peroxisomal volume fraction may increase over 20-fold during subsequent growth (18). In conventional degradation experiments, cells have been used that were in the late exponential growth phase and, thus, had accumulated numerous peroxisomes. Because peroxisomes in *H. polymorpha* are degraded individually and sequentially, the relative high numbers of peroxisomes in such cells in fact could mask the rapid degradation of Pex3p from the relatively few organelles that are

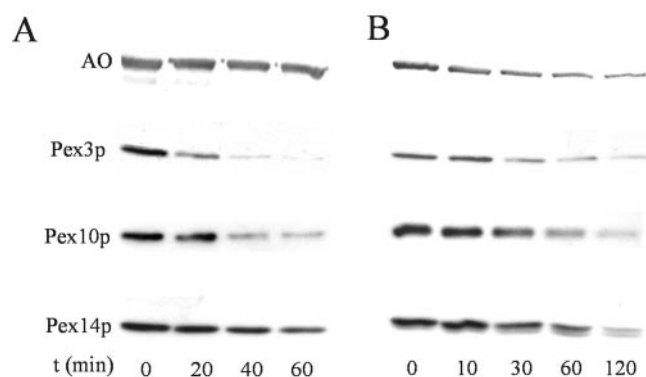


FIG. 2. Kinetics of selective peroxisome degradation in WT *H. polymorpha* in relation to the cultivation conditions. Selective peroxisome degradation was induced in methanol-grown cells at the early exponential growth phase ($OD_{663} = 0.8$) (A) or late exponential growth phase ($OD_{663} = 2.0$) (B) by the addition of glucose. Samples were taken at regular time intervals after the addition of glucose ($t = 0$ h). Samples corresponding to equal volumes of the cultures were loaded per lane. Blots were decorated with α -AO, α -Pex3p, α -Pex10p, and α -Pex14p antibodies. In cells from the early exponential growth phase (A), Pex3p levels dropped rapidly compared with AO and Pex14p. This difference is much less clear when glucose was added to methanol-grown cells at the late exponential growth phase (B).

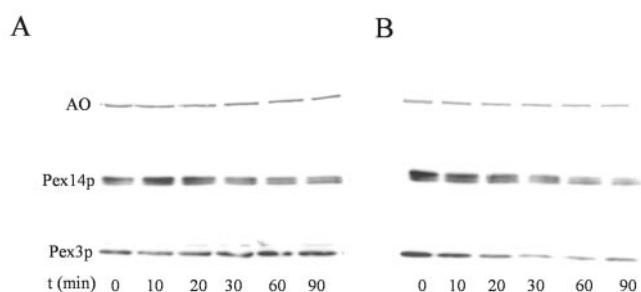


FIG. 3. The proteasome inhibitor MG-132 blocks Pex3p degradation. WT *H. polymorpha* cells were grown on methanol medium until the mid-exponential growth phase. Cells were collected at 0, 10, 20, 30, 60, and 90 min upon the addition of glucose in the presence (A) or absence (B) of the proteasome inhibitor MG-132. Western blots were decorated with antibodies against AO, Pex14p, and Pex3p. In the presence of the proteasome inhibitor (A), degradation of Pex3p is completely abolished as well as Pex14p and AO degradation. In the absence of MG-132 (B), these proteins are normally degraded.

tagged for degradation at a certain stage of macropexophagy.

Therefore, we decided to analyze the macropexophagy process in cells that contained only few peroxisomes and compared the data with those obtained in cells from the late exponential growth phase in which they contained numerous peroxisomes. The results are depicted in Fig. 2. Upon glucose-induced macropexophagy in cells grown to an optical density of 0.8 ($OD_{663} = 0.8$), Pex3p is rapidly degraded relative to AO and Pex14p (Fig. 2A). A similar experiment performed on cells grown to $A_{663} = 2.0$ gave essentially identical results: the kinetics of Pex3p degradation are faster than those of AO and Pex14p (Fig. 2B). However, in this experiment, Pex3p is still detectable 120 min after the shift to glucose medium, whereas the protein is almost undetectable already within 60 min in case of early exponential cells ($OD_{663} = 0.8$). Thus, the proper visualization of relatively rapid Pex3p reduction on Western blots appears to be related to the growth phase of the cells and thus ultimate peroxisome numbers.

Pex3p Degradation Is Prevented in the Presence of the Proteasome Inhibitor MG-132—A possible option for an alternative mode of Pex3p degradation would be degradation by the pro-

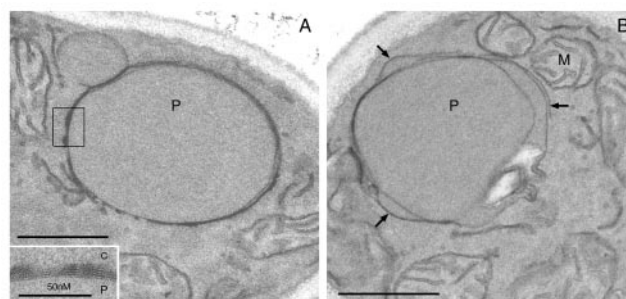


FIG. 4. The proteasome inhibitor MG-132 interferes with pexophagy. The first stage in macropexophagy is sequestration of individual peroxisomes by membrane layers. A, a typical example of a sequestered peroxisome in methanol-grown WT *H. polymorpha* cells shifted for 60 min to glucose-excess conditions in the absence of MG-132. The peroxisome is completely surrounded by several membrane layers. Inset, a magnification of the boxed area. In the presence of MG-132, membranes are formed as well, but these fail to closely envelop (arrows) the organelle (B). Ultrathin sections of $KMnO_4$ -fixed cells are shown. The bar represents 0.5 μ m. C, cytosol; M, mitochondrion; P, peroxisome.

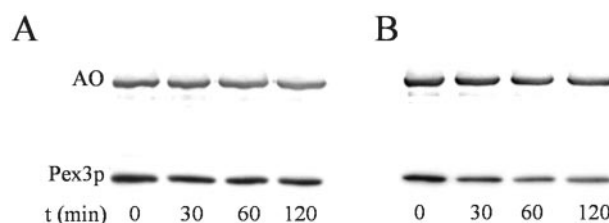


FIG. 5. ScPex3p and fusion of GFP to HpPex3p affect macropexophagy. Degradation of *S. cerevisiae* Pex3p produced in *H. polymorpha* pex3 ($pex3::P_{PEX3}ScPEX3$) cells was studied by Western blot analysis upon the addition of glucose to methanol-grown cells (A). The data show that the levels of ScPex3p and AO remained constant in time indicating that peroxisome degradation is impaired. In cells in which GFP was fused to the C terminus of Pex3p, degradation of Pex3p and AO was strongly retarded as is evident from the slight decrease in the Pex3p and AO levels detected with time (B). Samples were collected 0, 30, 60, and 90 min following the addition of glucose to cultures on methanol. Samples corresponding to equal volumes of the cultures were loaded per lane. Blots were decorated using antibodies against ScPex3p, HpPex3p, or AO.

teasome in the cytosol. To analyze this, experiments were performed in the presence of a proteasome inhibitor. To this purpose, 50 μ M MG-132 was added to a batch culture of *H. polymorpha* in the exponential growth stage on methanol ($OD_{663} = 1.3$) prior to the addition of excess glucose to induce macropexophagy. The results (Fig. 3A) unequivocally show that the initial rapid degradation of Pex3p observed in control cultures lacking MG-132 (Fig. 3B) is prevented. Also, the decrease in Pex14p and AO protein levels was retarded (Fig. 3A) relative to that in control cultures without proteasome inhibitor (Fig. 3B).

Electron microscopical analysis confirmed that peroxisomes were not degraded in the presence of MG-132. In these cells, the peroxisome degradation process appeared to be disturbed at an early stage, namely sequestration. As expected, during normal pexophagy in the absence of MG-132, individual peroxisomes are sequestered from the cytosol by several membrane layers (Fig. 4A). Also, in the presence of MG-132, additional membranes were formed. However, they failed to fully and closely envelop the organelles (Fig. 4B).

ScPex3p and Fusion of Green Fluorescent Protein to HpPex3p Affect Macropexophagy—We next analyzed whether the authentic *H. polymorpha* Pex3p was required to allow macropexophagy to occur. To this end, we analyzed peroxisome

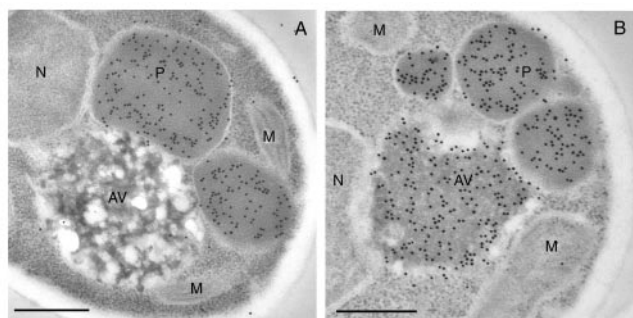


FIG. 6. Peroxisome degradation is prevented in *H. polymorpha* *pex3::P_{P_{PEX3}}ScPEX3*. Immunocytochemical localization of AO protein in methanol-grown cells 60 min after the addition of glucose. In *H. polymorpha* cells in which the *HpPEX3* gene has been replaced by the *S. cerevisiae* *PEX3* gene (*pex3::P_{P_{PEX3}}ScPEX3*), anti-AO-dependent labeling is only observed on peroxisomes and is invariably absent on vacuoles, confirming that peroxisomes are not degraded in these cells (A). In WT control cells, AO labeling is observed both on peroxisome and vacuole profiles, indicative for the ongoing peroxisome degradation (B). Ultra-thin sections of glutaraldehyde-fixed cells labeled with anti-AO antibodies are shown. The bar represents 0.5 μ m. AV, autophagic vacuole; M, mitochondrion; N, nucleus; P, peroxisome.

degradation in an available *H. polymorpha* *pex3* strain that was functionally complemented by the *Saccharomyces cerevisiae* *PEX3* gene under control of the endogenous *H. polymorpha* *PEX3* promoter, *pex3::P_{P_{PEX3}}ScPEX3*. This strain can normally grow on methanol and displays normal WT properties in peroxisome biogenesis and function (19). However, after a shift of methanol-grown cells of this strain to excess glucose conditions, the level of ScPex3p did not decrease. Moreover, also the level of AO remained unaltered, indicating that peroxisome degradation did not occur (Fig. 5A). Electron microscopical analysis confirmed that the ScPex3p containing peroxisomes were not degraded in these cells (Fig. 6). In WT controls, a significant anti-AO-dependent labeling was found both on peroxisome and vacuole profiles 60 min after the shift of cells to glucose, a phenomenon indicative for vacuolar degradation of peroxisomes. However, in *pex3::P_{P_{PEX3}}ScPEX3* cells shifted to glucose excess conditions and anti-AO labeling was confined to peroxisomes, whereas vacuoles were invariably devoid of anti-AO labeling (Fig. 6).

Fusion of Green Fluorescent Protein (GFP)¹ to the extreme C terminus of *H. polymorpha* Pex3p also strongly interfered with macropexophagy. In a conventional degradation experiment, the levels of HpPex3p-GFP and AO decreased much slower (Fig. 5B) compared with Pex3p and AO in WT cells. Apparently, the GFP tag interferes with the rapid degradation of Pex3p and the subsequent turnover of the organelle.

Rapid Pex3p Degradation Does Not Occur during Normal Growth of Cells on Methanol—To test whether Pex3p degradation is a rapid constitutive process and also occurs under conditions where peroxisome degradation is not induced, we tested the effect of the proteasome inhibitor MG-132 on Pex3p levels in cultures that were in the exponential growth phase on methanol. As shown in Fig. 7, the addition of MG-132 to such cells did not result in an increase in Pex3p levels relative to controls that lack MG-132. These data indicate that at normal growth conditions, no strong continuous Pex3p turnover occurs. Instead, they strongly support the notion that the decline of Pex3p upon initiation of pexophagy is the result of induced Pex3p degradation rather than a shift in equilibrium of Pex3p

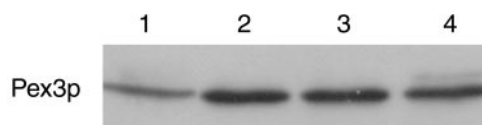


FIG. 7. Kinetics of Pex3p levels in WT *H. polymorpha*. Cells were shifted from glucose to methanol, and after incubation for 17 h, cells were incubated for 2 h in the presence or absence of MG-132. Blots were decorated with antibodies against Pex3p. Lane 1, glucose-grown cells; lane 2, 17-h methanol; lane 3, -MG-132; and lane 4, +MG-132. Equal amounts of cells were loaded per lane.

synthesis, which is reduced because of the addition of glucose, and continuous degradation.

DISCUSSION

In this paper, we provide evidence that upon the induction of selective degradation of peroxisomes (macropexophagy) in *Hansenula polymorpha* a peroxisomal membrane protein, Pex3p, does not follow the normal degradative pathway of other peroxisomal constituents in the vacuole.

Pex3p is one of the first identified peroxisomal membrane proteins and has been observed in various organisms including man (7, 20–23). Recently, a Zellweger syndrome patient belonging to complementation group G was shown to be affected in Pex3p function (24). In *H. polymorpha*, the protein is essential for peroxisome biogenesis and maintenance (7). It was first described in *S. cerevisiae* (20) as an integral component of the membrane that spans the membrane once with the N terminus inside the organelle and with the C terminus exposed to the cytosol. However, in *H. polymorpha*, Pex3p appears to be associated with the outer surface of the peroxisomal membrane without spanning it (25).

Selective turnover of membrane proteins has been observed in various cellular membranes. However, for peroxisomal membranes, this process has not been described before. So far, three mechanisms are known that result in the specific degradation of selected membrane proteins. Proteins of the plasma membrane (e.g. yeast Ste6p) first undergo ubiquitin-mediated internalization (endocytosis). Subsequently, the protein is transported via vesicle trafficking to the vacuole where it is degraded (26, 27). In the endoplasmic reticulum, membrane proteins to be degraded are marked with ubiquitin as well but subsequently are dislocated and degraded by the proteasome in the cytosol (28, 29). A well known example of this process is the degradation of defective cystic fibrosis transmembrane conductance regulator (a cAMP-regulated chloride channel), which leads to reduced lung function and, ultimately, death (30).

A third membrane protein degradation pathway occurs in bacteria, mitochondria, and chloroplasts and involves membrane-bound AAA-proteases that remove proteins from the membrane and subsequently degrade them (31). So far no peroxisomal membrane-bound proteases have been identified. However, general cellular degradation mechanisms like the ubiquitin/proteasome system may play a role in degradation of peroxisomal membrane proteins as well.

In *H. polymorpha*, peroxisomes are degraded individually, a process that is not inhibited by cycloheximide and apparently does not require protein synthesis. In *P. pastoris*, an identical process has been observed upon exposure of methanol-grown cells to excess ethanol (4). In *P. pastoris* but not in *H. polymorpha*, a second mode of pexophagy is also described, namely the glucose-induced bulk turnover of peroxisome clusters. This process designated microautophagy is dependent on protein synthesis (4).

At present, the first clues of the principles of the macro/micropexophagy machinery in yeast become clear. Taking to

¹ The abbreviations used are: GFP, green fluorescent protein; AO, alcohol oxidase; WT, wild type.

gether the data of *S. cerevisiae*, *H. polymorpha*, and *P. pastoris*, the picture emerges that these processes overlap with other vacuolar sorting pathways, e.g. endocytosis, macroautophagy, and microautophagy and the cytoplasm to vacuole transport pathway (32–34). However, the selectivity of macropexophagy in *H. polymorpha* is apparently organized at the initial stages of the process and most probably is not overlapping with other vacuolar protein sorting pathways (35, 36).

The selectivity of peroxisome degradation during macropexophagy in *H. polymorpha* is manifested by the close sequestration of organelles to be degraded by various membrane layers prior to their uptake in the vacuole. Crucial for the understanding of the function of Pex3p removal in this process is information on how and at which stage of the process the protein is degraded. To this purpose, we analyzed two *H. polymorpha* *pdd* mutants, namely *pdd1*, which is known to be affected in the first step of the pexophagy process, organelle sequestration, and *pdd2*, which shows normal sequestration but is impaired in the vacuole fusion process (15). Upon induction of macropexophagy, the turnover of Pex3p was fully prevented in *pdd1* cells (14) but not in *pdd2* cells. This led us to conclude that Pex3p degradation occurs prior to or during sequestration of peroxisomes. Apparently, Pex3p is not degraded in the vacuole but in the cytosol, a process that may directly or indirectly involve the function of the proteasome, because inhibition of the proteasome activity by MG-132 prevented Pex3p turnover.

The removal of Pex3p from the peroxisomal membrane seems to be a prerequisite to allow macropexophagy to proceed and requires the WT *H. polymorpha* Pex3p. Indicative for this hypothesis are the findings that the replacement of HpPex3p by the bakers' yeast homologue, ScPex3p, did not affect peroxisome biogenesis but inhibited organelle degradation. When GFP was fused to the C terminus of HpPex3p degradation of Pex3p-GFP and peroxisome turnover was significantly retarded. Possibly, correct exposure of specific domains in HpPex3p is important for the removal of the protein, a process that might be disturbed upon fusion of GFP to the C terminus. Together with the observation that initiation of macropexophagy does not require protein synthesis, this finding lends support to the view that probably modification/conformational changes or rearrangement reactions of already existing proteins have to occur to allow initiation of this degradation process. We speculate that the presence of GFP at the extreme C terminus of Pex3p may interfere with recognition of the domain that is involved in the initial stage of macropexophagy and/or blocks conformational changes/modifications of the protein that are necessary to allow its degradation. The importance of Pex3p removal to initiate peroxisome degradation is also in line with our previous observation that in cells in which HpPex3p is overproduced, selective peroxisome degradation is disturbed (37). Four hours after the induction of peroxisome degradation in a HpPex3p-overproducing strain, HpPex3p was still present, whereas in the WT control, HpPex3p was not detectable anymore at that time point (37). These data also indicate that the presence of HpPex3p inhibits peroxisome degradation.

Previously, we showed that Pex14p, one of the main docking proteins in the peroxisomal matrix protein import pathway, is essential to allow macropexophagy (5). The information that governed this process appeared to be located in the extreme N terminus of the protein (6). The data suggested that Pex14p may act as a kind of molecular switch that discriminated between organelle biogenesis and susceptibility to organelle degradation. Yet, it is unclear whether Pex3p and Pex14p take part in a joint machinery (e.g. in the tagging process of organelles destined for degradation) or serve completely different

roles in the degradation process.

A clue in the order of Pex14p function and Pex3p removal may be deduced from the experiments in cells that lack Pex14p but in which peroxisome formation was restored by overproduction of the PTS1 receptor, Pex5p (strain *pex14::P_{AOX}PEX5^{mc}*). The reintroduction of the organelles allowed us to address the fate of Pex3p upon exposure of the cells to excess glucose. Our data revealed that upon induction of macropexophagy in methanol-induced *pex14::P_{AOX}PEX5^{mc}* cells the level of Pex3p decreased with time. Thus, Pex3p is degraded independent from the function of Pex14p in macropexophagy. Essentially, similar experiments were performed with a strain that produced a truncated form of Pex14p that lacked the initial 64 N-terminal amino acids (PEX14-ΔN64) and thus could not degrade peroxisomes (6). Because in cells of the PEX14-ΔN64 strain Pex3p was degraded, we assume that in macropexophagy, the degradation of Pex3p occurs prior to the step that requires (the N terminus of) Pex14p.

In conclusion: this work has extended the list of multiple functions of Pex3p in peroxisome development (matrix protein import, membrane biogenesis, maintenance, and stabilization) to a role in peroxisome degradation. The molecular mechanisms of how the protein can serve these various mechanisms is largely unclear and is a topic of current investigations.

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